Supplementary Information

Circulating Mutant DNA to Assess Tumor Dynamics

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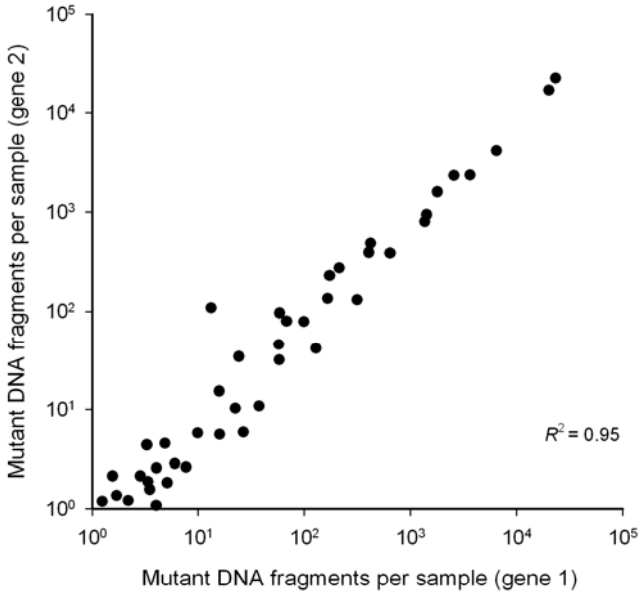
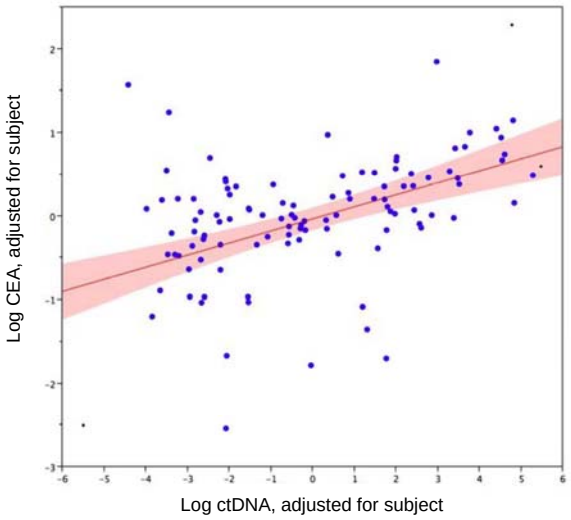
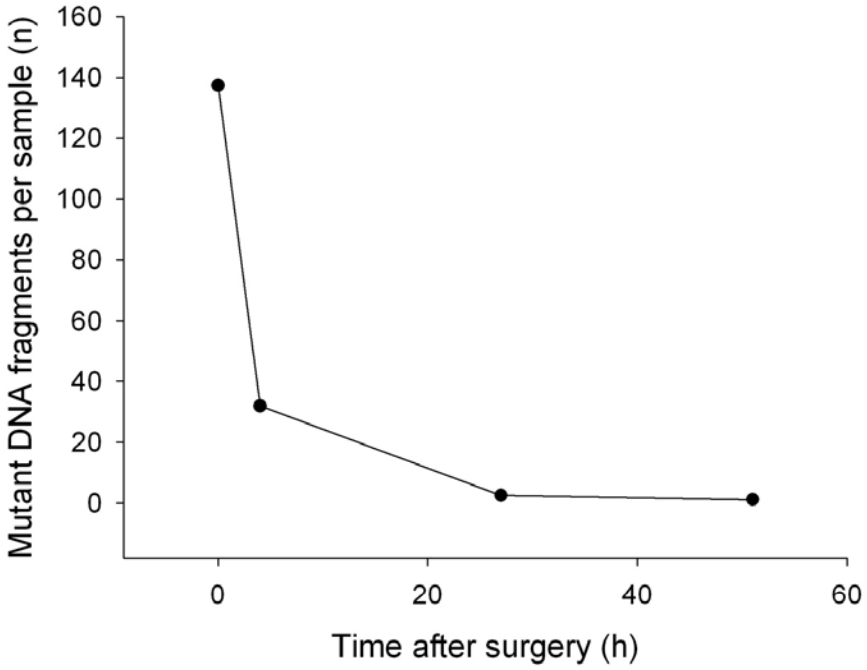


Supplementary Fig. 1a Scatter plot of the ctDNA levels determined from two independent BEAMing assays on two distinct mutations from the same patients. The correlation coefficient was R^2=0.95.



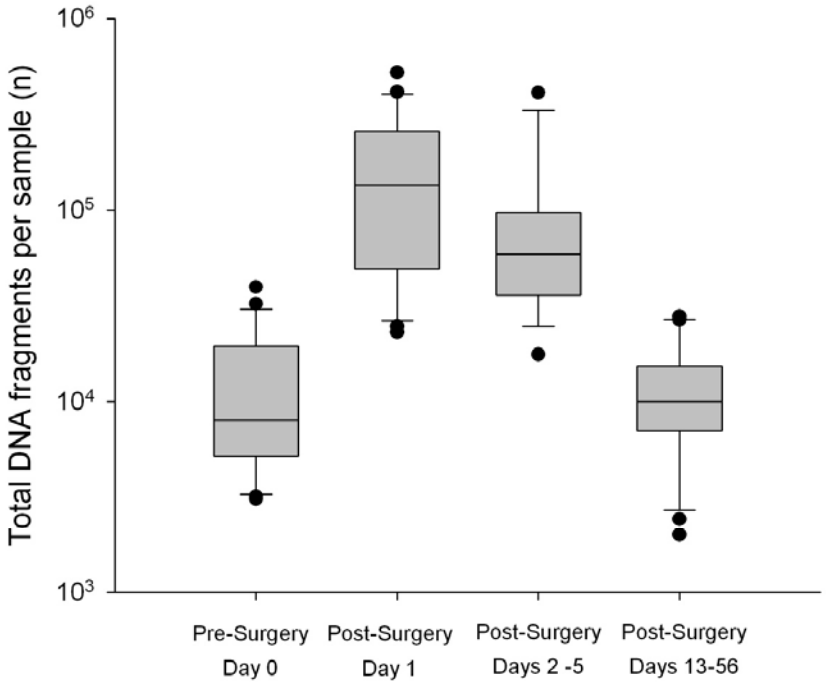
Supplementary Fig. 1b Comparisons between plasma CEA and ctDNA levels in the same plasma samples. A partial residual plot comparing CEA and ctDNA levels, corrected for individual clustering, is shown. All subjects' CEA and ctDNA values were used for this comparison. There was a modest overall correlation between CEA levels and ctDNA after correcting for clustering within subjects (R^2 = 0.2, P<0.001).

Supplementary Fig. 2



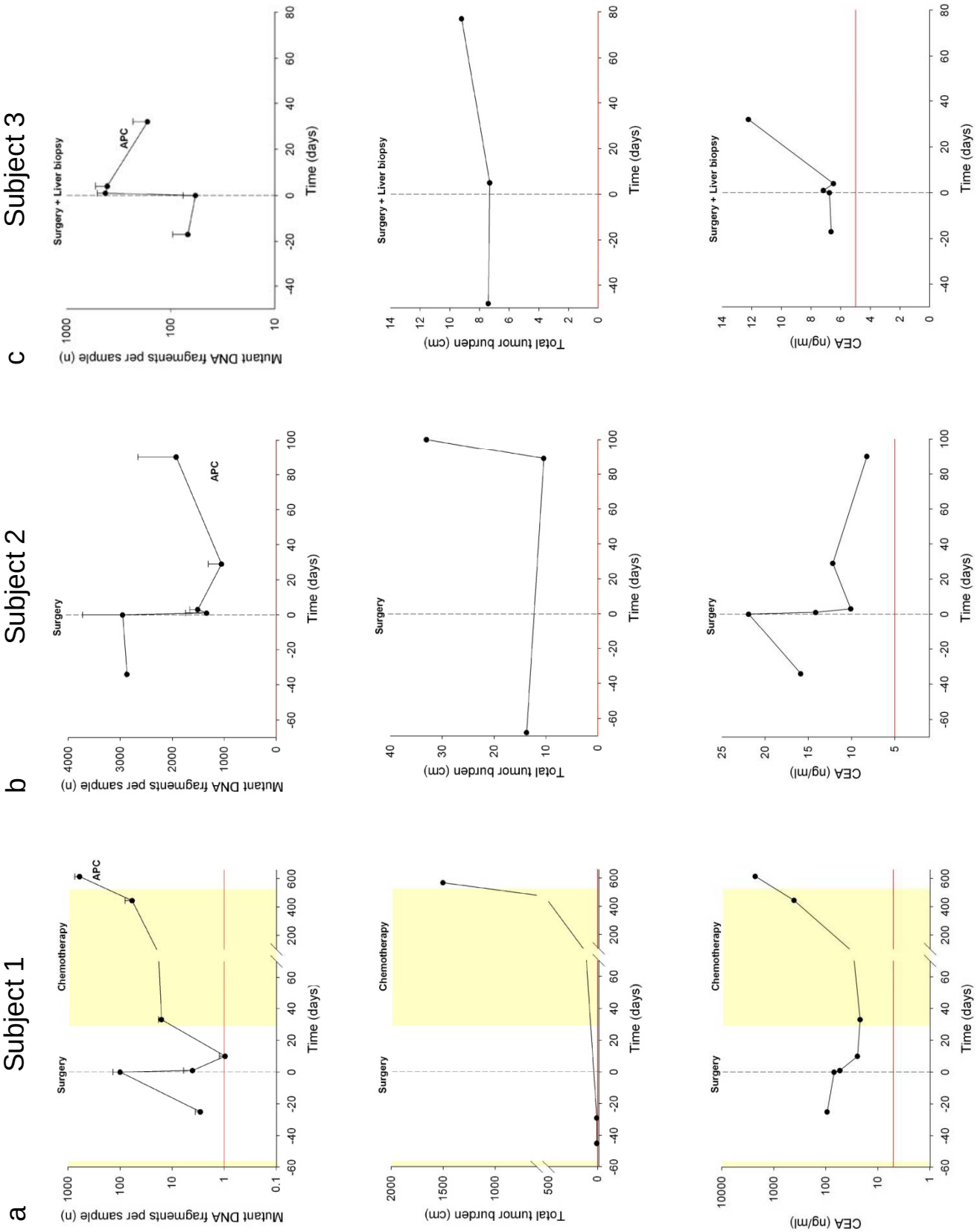
Supplementary Fig. 2 ctDNA half-life. The y axis represents the level of ctDNA in the plasma of subject 9. The x axis represents the time from resection, with zero as the time of tumor removal. To calculate the half-life, a curve fit ($f(t) = a^{-\lambda t}$) based on the Marquardt-Levenberg algorithm was performed, yielding a half-life of 114 min.

Supplementary Fig. 3



Supplementary Fig. 3 Total DNA fragments in plasma prior and after surgery. The Wisker box plot shows the total number of DNA fragments in 2 ml plasma, estimated by real-time PCR at baseline (day 0), post-surgery (day 1), day of discharge (days 2-5), and at the 1st Follow up (days 13-56).

Supplementary Fig. 4



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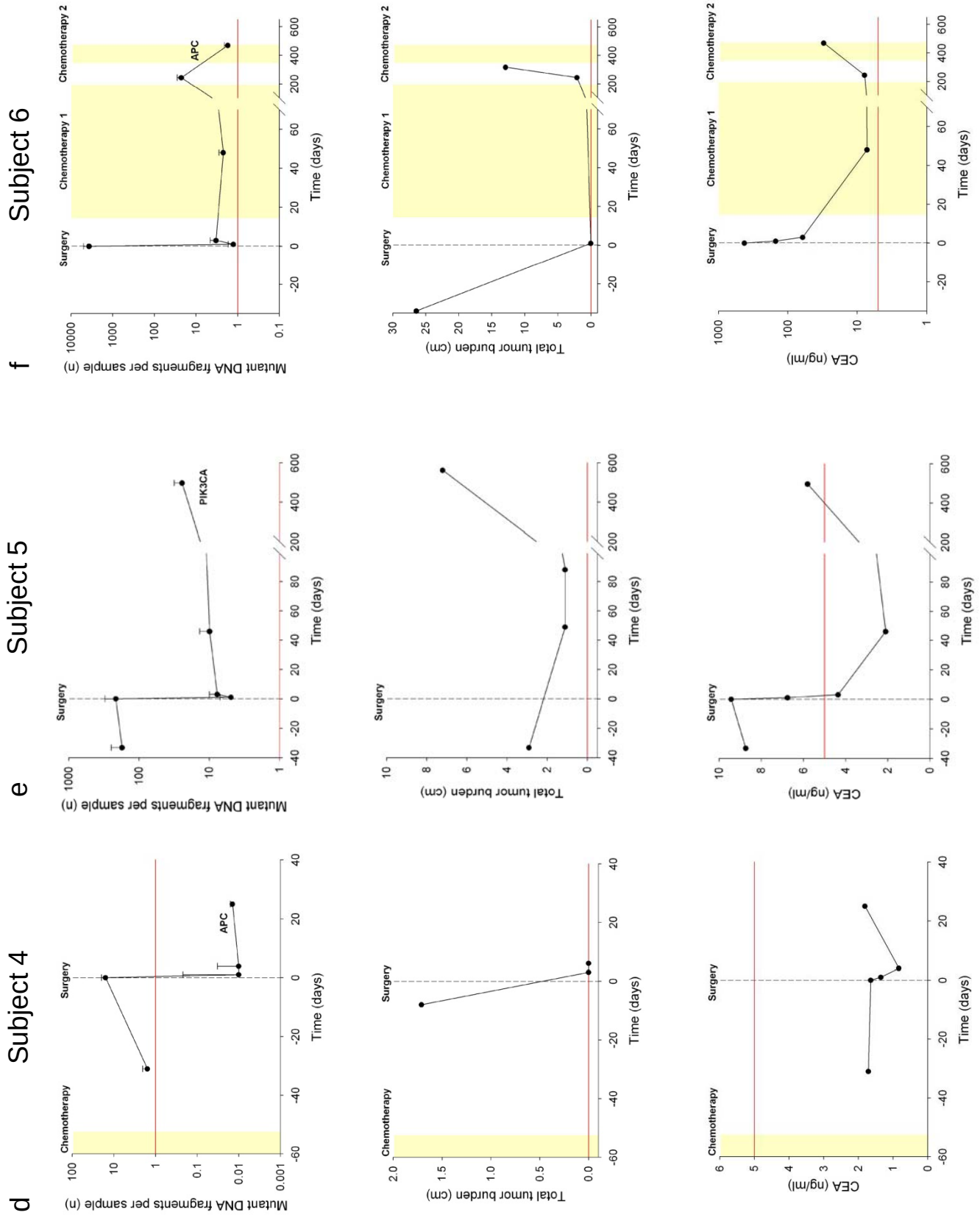

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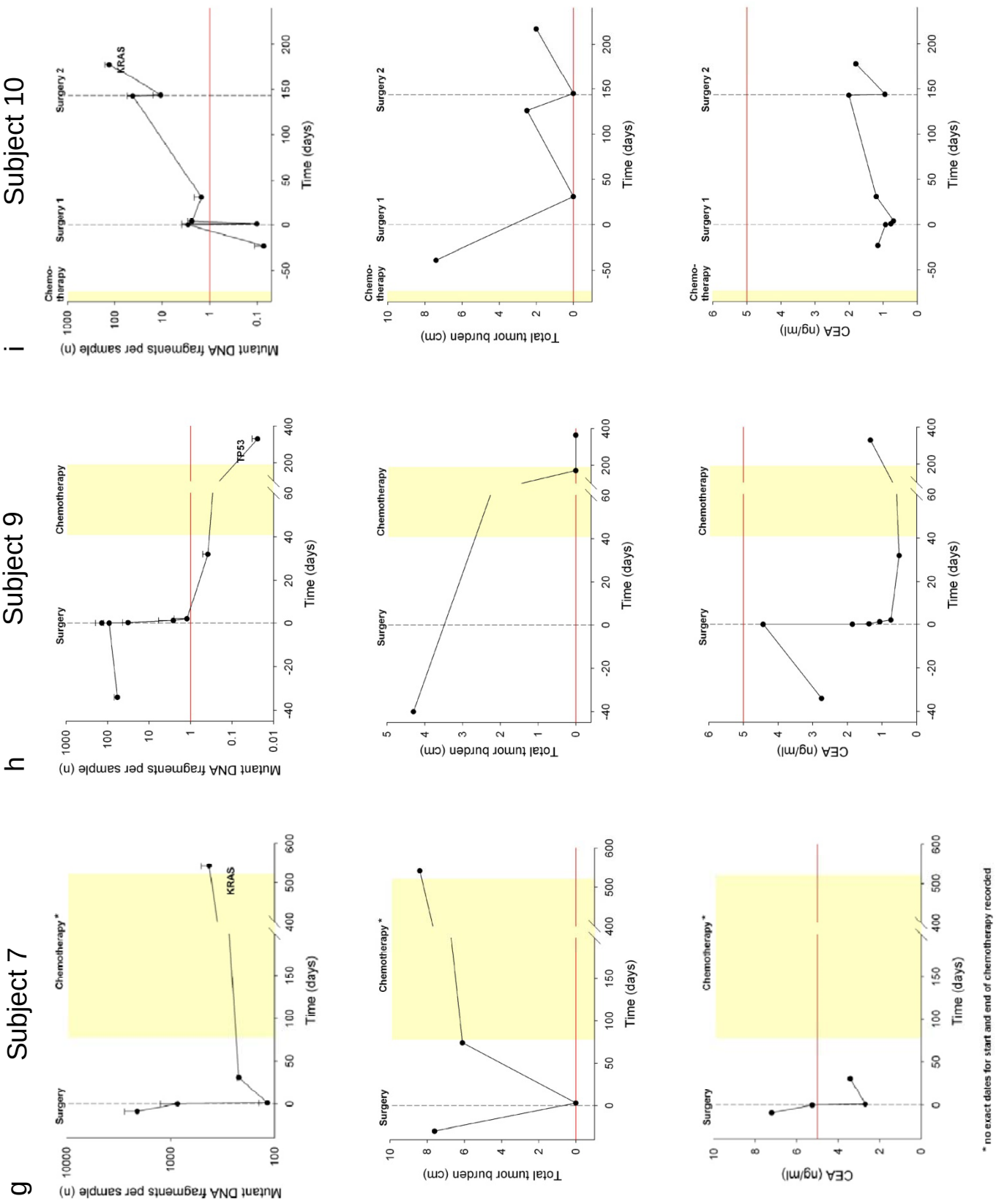

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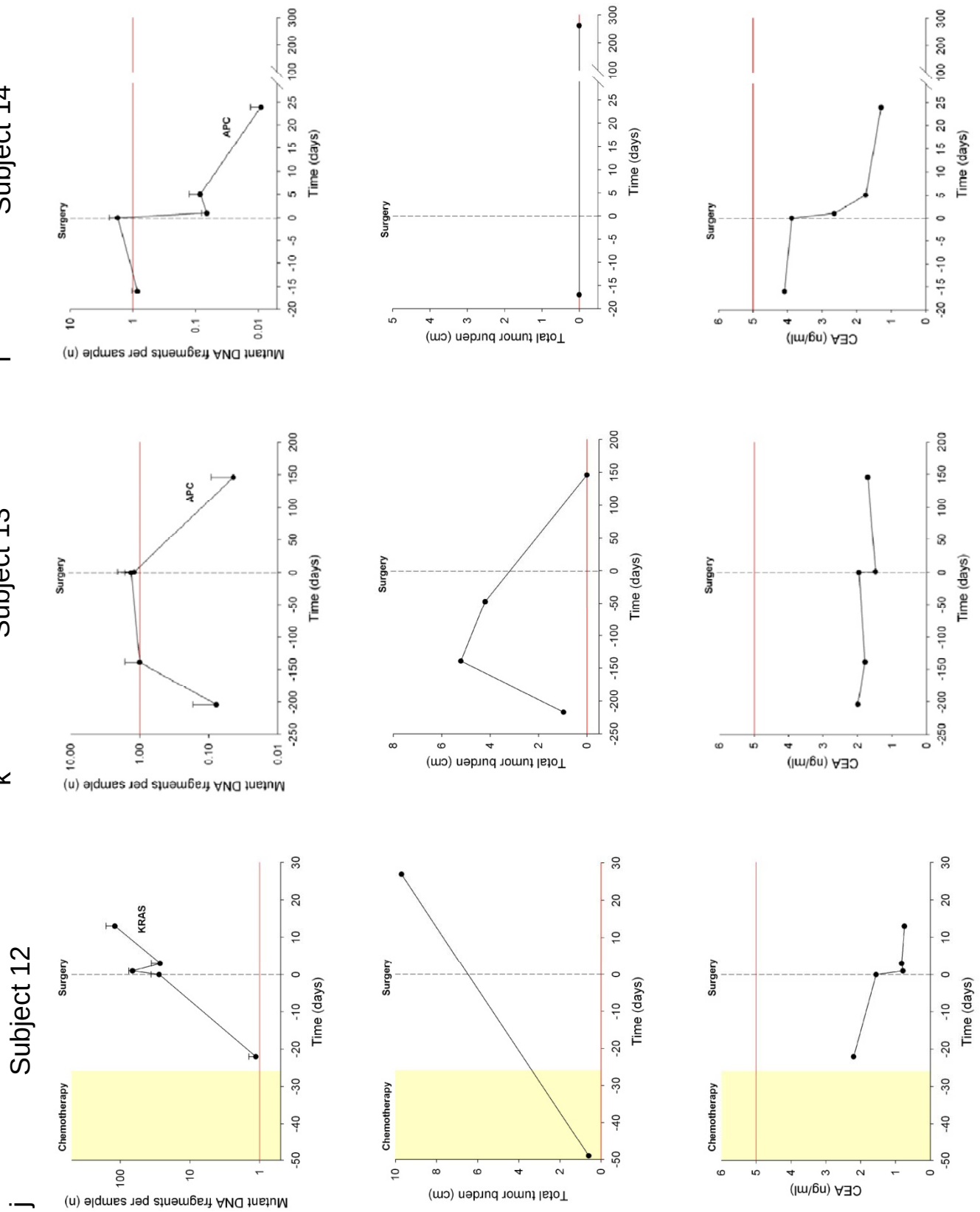

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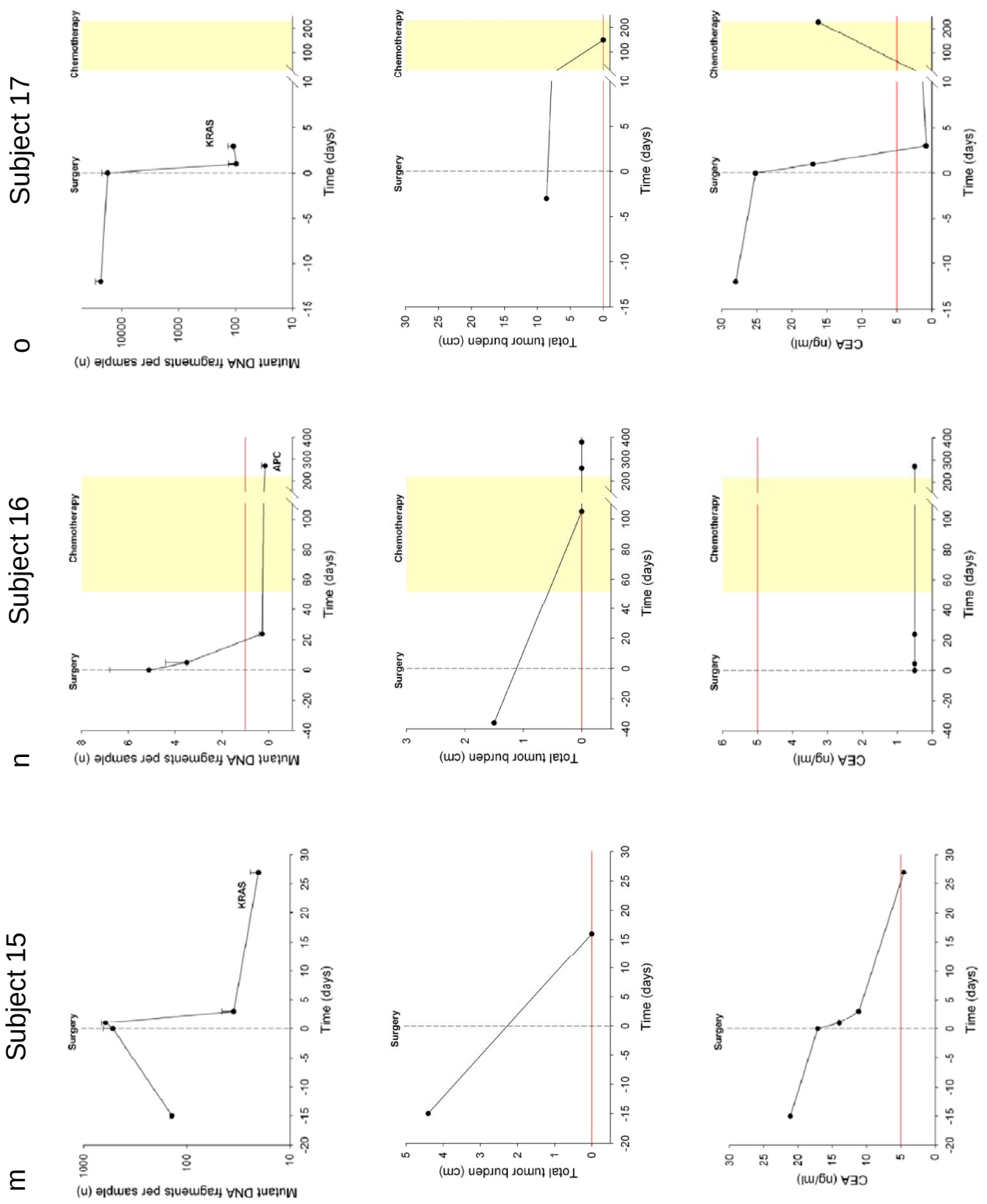

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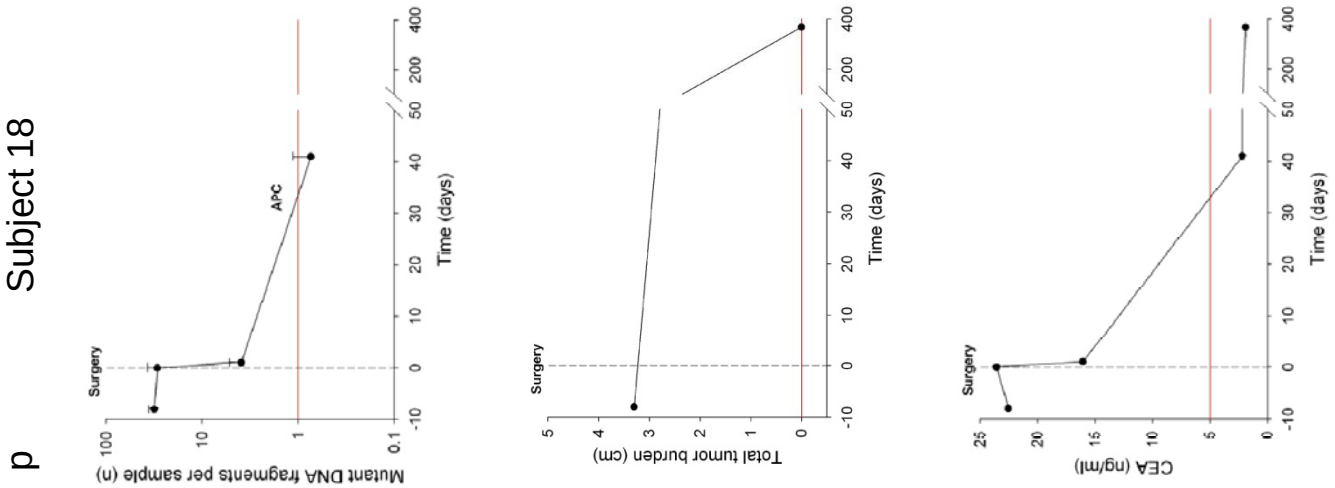


Supplementary Fig. 4 Individual subject summaries

Fig. 4a Patient 1 originally underwent a low anterior resection for rectal carcinoma and was found to have multiple liver metastases with PET/CT scanning. The patient received post-operatively 5-fluorouracil, oxaliplatin (FOLFOX) and bevacizumab (Chemotherapy) for two cycles and repeat imaging revealed a partial response. At the time of study entry, the patient underwent right hepatectomy and left lobe wedge resection and cholecystectomy (Surgery), followed by chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and bevacizumab (Chemotherapy). Repeat imaging revealed multiple new lung lesions and two new liver lesions. Various other chemotherapy regimens were utilized with continued progression of disease. The patient is currently being considered for a phase I trial.

Fig. 4b Patient 2 was originally diagnosed with a T3N0M0 colon adenocarcinoma and underwent a left hemicolectomy. At the time of study entry, the patient underwent a right hepatic lobectomy and partial diaphragm resection for metastatic disease (Surgery). Repeat imaging studies revealed progressive disease three months following liver lobectomy and the patient died of disease shortly thereafter.

Fig. 4c Patient 3 was initially found to have metastatic mucinous colon adenocarcinoma, T2N1M1 with a single liver metastasis who underwent a right hemicolectomy with planned liver resection (Surgery). However, the patient was found to have diffuse peritoneal implants at the time of surgery, and the liver resection was not performed. Post-operative CT scans revealed evidence of progressive disease with enlarging liver lesion and a new pulmonary nodule. The patient opted to proceed with supportive care only and died of disease approximately one year following the surgery.

Fig. 4d Patient 4 was diagnosed with metastatic colon adenocarcinoma. At study entry, 12 months following the initial surgery, the patient received pre-operative chemotherapy with 5-fluorouracil, oxaliplatin and bevacizumab. The patient then underwent a partial hepatectomy of two liver lesions with radio-frequency ablation of the margins with pathology concurrent with recurrent metastatic adenocarcinoma (Surgery). Subsequent CT scans have revealed no evidence of disease recurrence to date.

Fig. 4e Patient 5 was diagnosed with rectal adenocarcinoma. The patient underwent a left hepatectomy for recurrent disease at the time of entry into the study (day zero). Except for a questionable lung nodule in the left upper lobe lung, there was no evidence of disease immediately after surgery. Fifteen months later, disease recurrence was noted, with lesions found in both liver and lung.

Fig. 4f Patient 6 originally presented with a T3N1M1 colon adenocarcinoma, and at the time of study entry underwent a right hepatectomy and right lower lobe lung wedge resection (Surgery). Follow-up CT scans revealed no evidence of disease and the patient was started on chemotherapy. Eight months later, repeat imaging then revealed a new liver metastasis. The patient then switched to irinotecan, 5-fluorouracil and bevacizumab (Chemotherapy 1), but despite four months of therapy still had persistent disease on follow-up CT scans. They were subsequently started on 5-fluorouracil, leucovorin, oxaliplatin and bevacizumab (Chemotherapy 2**)**

Fig. 4g Patient 7 has a prior history of a resected T3N2M1 rectosigmoid adenocarcinoma. At the time of study entry, the patient underwent surgical excision of two recurrent liver lesions, and an additional 4 liver lesions were treated with radiofrequency ablation (Surgery**)**. Post-operative imaging revealed no evidence of disease, however, imaging three months later revealed new liver disease and new lung metastases. The patient was started on irinotecan, cetuximab, and bevacizumab (Chemotherapy). Despite chemotherapy, on follow-up imaging the patient was noted to have persistent and progressing disease.

Fig. 4h Patient 9 originally presented with a T3N1M0 colon adenocarcinoma followed by adjuvant 5-fluorouracil and leucovorin. At the time of study entry, a solitary liver lesion was noted, and the patient underwent a right hepatectomy, with pathology revealing recurrent adenocarcinoma (Surgery). The patient was given post-operative 5-fluorouracil , oxaliplatin and bevacizumab (Chemotherapy) and follow up imaging has revealed no evidence of disease recurrence, with evidence of a fully regenerated liver.

Fig. 4i Patient 10 was originally diagnosed with metastatic colorectal adenocarcinoma to the liver and was treated with 5-fluorouracil, oxaliplatin and bevacizumab for four months (Chemotherapy). A right hepatectomy and right hemicolectomy was performed (Surgery 1). The liver resection was margin positive. Post-operative imaging revealed no evidence of disease. Repeat imaging performed three months later revealed 3 new left liver lesions and the patient subsequently underwent a left liver hepatectomy with radio-frequency ablation to the margins (Surgery 2). Post-operative imaging revealed no evidence of disease. At two months follow-up the patient was found to have boney metastases with a T7 compression fracture for which the patient underwent external beam radiation.

Fig. 4j Patient 12 was initially diagnosed with metastatic colon adenocarcinoma. At the time of study entry, the patient underwent a repeat partial hepatectomy with radio-frequency ablation (Surgery) after achieving some stabilization of disease with 5-fluorouracil, leucovorin, and oxaliplatin (Chemotherapy). Post-operative scans revealed no evidence of disease in the liver. However, CT scan of the chest revealed numerous new pulmonary lesions and a follow up PET showed new liver lesions as well. The patient was then referred for a Phase I clinical study.

Fig. 4k Patient 13 had a history of metastatic colon cancer resected from the sigmoid colon, liver and xiphoid process. Approximately 14 months after their original diagnosis, a CT scan revealed a 1 cm lesion in the liver, and a follow-up PET scan showed two adjacent foci of disease near the left hepatic lobe. A CT scans performed three months later showed increase in size of the hepatic lesions and a new peritoneal implant. They then underwent resection of the recurrent disease with partial hepatectomy, partial gastrectomy, and partial omentectomy (Surgery). Follow-up CT scans performed 1-year following surgery showed hepatic and omental recurrences.

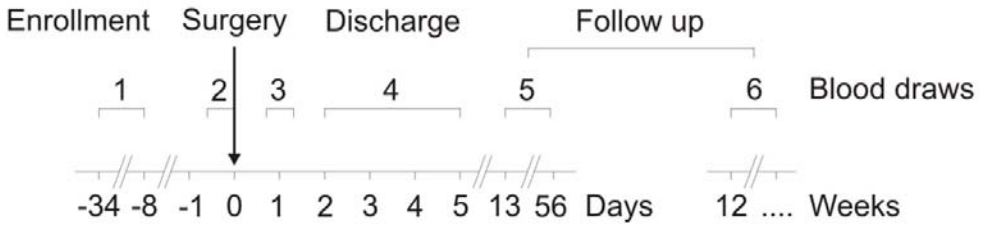
Fig. 4l Patient 14 was found to have colon adenocarcinoma on screening colonoscopy with CT scans showing no evidence of distant metastases. The patient underwent a sigmoid colectomy (Surgery) and pathology revealed a T3N0M0 tumor. No adjuvant chemotherapy was given and the patient was followed with serial CT scans. The last CT scan showed no evidence of disease.

Fig. 4m Patient 15 had a history of a completely resected T3N1Mx cecal mass and resected umbilical recurrence. Three years after the resection of the primary tumor, a CT scan of the abdomen then revealed a solitary liver metastasis. The patient underwent a right liver hepatectomy (Surgery). A follow-up CT scans one month later showed no evidence of disease, but the patient died of disease approximately one year later from recurrent metastatic disease.

Fig. 4n Patient 16 had a rectosigmoid mass on CT after being worked up for bright red blood per rectum, and underwent a sigmoid colectomy (Surgery). The patient was started on 5-fluorouracil, leucovorin and oxaliplatin, which was continued for the next five months (Chemotherapy). Follow-up CT scans following completion of therapy has shown no evidence of disease recurrence.

Fig. 4o Patient 17 is a patient with a history of resected colorectal cancer that was found by PET CT to have an isolated liver metastasis in the right lobe. They underwent a right hepatectomy (Surgery) and received post-operative chemotherapy with 5-fluorouracil, leucovorin, oxaliplatin and bevacizumab (Chemotherapy). They were found to have a recurrence 7 months after surgery.

Fig. 4p Patient 18 was found to have a T3N1Mx adenocarcinoma after undergoing a low anterior resection for a rectal mass. Three years later the patient was noted to have a left hepatic lobe lesion discovered on CT scan imaging. The patient underwent a laparoscopic liver resection (Surgery). They received no additional chemotherapy and are currently disease-free.



Supplementary Fig. 5 Plasma collection time-line.

Supplementary Table 1. Characteristics of study subjects.

Characteristic	Value (N=18)
Age - years	
Mean	59.8
Range	35-82
Sex	
Male	8 (44%)
Female	10 (56%)
Stage	
IV	16 (88%)
III	1 (6%)
II	1 (6%)
Differentiation	
Well	3 (17%)
Moderate	11 (61%)
Poor	2 (11%)
Unspecified	2 (11%)
Location of Metastases	
Liver	15
Lung	2
Omentum or peritoneal	2
Number of Surgeries	
1	15
2	2
3	1
Preoperative CEA (ng/ml)	
Mean	42
Range	0.5 - 2,250
% above normal range (>5 ng/ml)	55%

Supplementary Table 2. Mutations identified in tumor tissue.

Patient No.	Gene	Mutation (codon)	Plasma analyzed
1	APC	4461del t (1487)	Yes
	KRAS	G38A (13)	Yes
	TP53	C817T (273)	No
2	APC	C4031A (1344)	Yes
3	APC	C4348T (1450)	Yes
	KRAS	G35C (12)	Yes
	TP53	G733A (245)	No
4	APC	4465-4468del TTAC (1489)	Yes
	KRAS	G38A (13)	Yes
	TP53	G730A (244)	No
5	PIK3CA	G1624A (542)	Yes
	TP53	C844T (282)	Yes
6	APC	G4189T (1397)	Yes
	KRAS	G35A (12)	Yes
	TP53	G743A (248)	No
7	KRAS	G35T (12)	Yes
8	PIK3CA	G1624A (542)	Yes
	TP53	G818A (273)	Yes
9	TP53	C535T (179)	Yes
10	APC	C4067A (1356)	Yes
	KRAS	G35A (12)	Yes
11	TP53	C817T (273)	Yes
12	PIK3CA	A3140G (1047)	No
	KRAS	G38A (13)	Yes
13	APC	G3862T (1288)	Yes
14	APC	3877-3889del ACACAGGAAGCAG	Yes
15	KRAS	G35T (12)	Yes
16	APC	3905del T (1302)	Yes
	TP53	C844T (282)	Yes
17	APC	C2626T (876)	Yes
	KRAS	G35A (12)	Yes
18	APC	C4012T (1338)	Yes

In cases where there was a limited amount of plasma available, we only evaluated one or two mutations and therefore did not design a BEAMing assay for every mutation identified.

Supplementary Methods

Isolation of DNA from formalin-fixed, paraffin embedded (FFPE) tumor tissue

Eighteen tumor specimens were collected after liver or colon surgery, fixed in formalin, and embedded in paraffin. Ten µm sections were cut and mounted on PEN-membrane slides (Palm). The sections were deparaffinized and stained with hematoxylin and eosin. All specimens underwent histological examination to confirm the presence of tumor tissue, which was dissected from completely dried sections with a MicroBeam laser microdissection instrument (Palm). The dissected tumor tissue was digested overnight at 60°C in 15 µl ATL buffer (Qiagen) and 10 µl Proteinase K (20 mg/ml; Invitrogen). DNA was isolated using the QIAamp DNA Micro Kit (Qiagen) following the manufacturer's protocol. The isolated DNA was quantified by hLINE-1 quantitative PCR as described below.

PCR amplification and direct sequencing of DNA isolated from tumor tissue

All DNA samples isolated from tumor tissue were analyzed for mutations in 26 regions of *APC* (19), one region of *KRAS* (1), two regions of *PIK3CA* (2), and four regions of *TP53* (4) using direct Sanger sequencing. Due to degradation of DNA in formalin-fixed and paraffin-embedded (FFPE) tissues, the amplicons were chosen to be between 74 to 132 bp in length. The first PCR was performed in a 10 µl reaction volume containing 50-100 genome equivalents (GEs) of template DNA (1 GE equals 3.3 pg of human genomic DNA), 0.5 U of Platinum Taq DNA Polymerase (Invitrogen), 1× PCR buffer (67 mM of Tris-HCl, pH 8.8, 67 mM of $MgCl_2$, 16.6 mM of $(NH_4)_2SO_4$, and 10 mM of 2-mercaptoethanol), 2 mM ATP, 6% (v/v) DMSO, 1 mM of each dNTP, and 0.2 µM of each primer. The sequences of the primer sets are listed in **Supplementary Methods Table 1**. The amplification was carried out under the following conditions: 94°C for 2 min; 3 cycles of 94°C for 15 s, 68°C for 30 s, 70°C for 15 s; 3 cycles of 94°C for 15 s, 65°C for 30 s, 70°C for 15 s, 3 cycles of 94°C for 15 s, 62°C for 30 s, 70°C for 15 s; 40 cycles of 94°C for 15 s, 59°C for 30 s, 70°C for 15 s. One microliter of the first amplification was then added to a second 10-µl PCR reaction mixture of the same makeup as the one described above, except that different primers were used (**Supplementary Methods Table 1)**. The second (nested) PCR reaction was temperature cycled using the following conditions: 2 min at 94°C; 15 cycles of 94°C for 15 s, 58°C for 30 s, 70°C for 15 s. The PCR products were purified using the AMpure system (Agencourt, Beverly, MA) and sequenced from both directions using BigDye Terminator v3.1 (Applied Biosystems). The primers used for sequencing had a 30 nucleotide (nt) polyT tag attached to the 5' prime end to improve the sequence quality at the beginning of the electrophoretogram (Tag1 primer: 5'-$(dT)_{30}$-tcccgcgaaattaatacgac-3'; M13 primer: 5'-$(dT)_{30}$-gtaaaacgacggccagt-3'). Sequencing reactions were resolved on an automated 96-capillary DNA sequencer (Spectrumedix). Data analysis was performed using Mutation Explorer (SoftGenetics).

Supplementary Methods Table 1 - Primers used for direct sequencing of DNA from tumor tissue

Amplicon No	Gene	Exon	PCR	Forward primer 5'-3'	Reverse primer 5'-3'
1	APC	5	1st	tgaagcaaggcaaatcagagt	tcgctgttttatcacttagaaacaa
			2nd	M13-caaatcagagttgcgatgga	Tag1-tcgctgttttatcacttagaaacaa
2	APC	6	1st	acataactaattaggtttcttgttttatttt	cctctgcttctgttgcttgg
			2nd	M13-acataactaattaggtttcttgttttatttt	Tag1-tgcttgggactgtaaaagctg
3	APC	15	1st	ggcaactaccatccagcaac	atctgggctgcagtggtg
			2nd	M13-tccagcaacagaaaatccag	Tag1-atctgggctgcagtggtg
4	APC	15	1st	tgtttctccatacaggtcacg	tggcttacattttgattaattccat
			2nd	M13-gtcacggggagccaatg	Tag1-tggcttacattttgattaattccat
5	APC	15	1st	tccaatatgtttttcaagatgtagttc	cagaatctgcttcctgtgtcg
			2nd	M13-tccaatatgtttttcaagatgtagttc	Tag1-tctgcttcctgtgtcgtctg
6	APC	15	1st	ctgaagatgaaataggatgtaatcagac	cttcagctgacctagttccaatc
			2nd	Tag1-ctgaagatgaaataggatgtaatcagac	M13-cttcagctgacctagttccaatc
7	APC	15	1st	cagattctgctaataccctgcaa	agggtgctgtgacactgctg
			2nd	Tag1-cagattctgctaataccctgcaa	M13-actgctggaacttcgctcac
8	APC	15	1st	ttggaactaggtcagctgaaga	gaagataaactagaaccctgcagtc
			2nd	Tag1-ttggaactaggtcagctSaaga	M13-gcagtctgctggatttggtt
9	APC	15	1st	gatcctgtgagcgaagttcc	tgcctggctgattctgaag
			2nd	M13-agcgaagttccagcagtgtc	Tag1-tgcctggctgattctgaag
10	APC	15	1st	cagcagactgcagggttctag	gtctgagcaccacttttggag
			2nd	M13-cagcagactgcagggttctag	Tag1-ccacttttggagggagatttc
11	APC	15	1st	tcttcaggagcgaaatctcc	gctaaacatgagtggggtctc
			2nd	Tag1-tcttcaggagcgaaatctcc	M13-atgagtggggtctcctgaac
12	APC	15	1st	ccaaaagtggtgctcagaca	caaaactatcaagtgaactgacagaag
			2nd	M13-gctcagacacccaaaagtcc	Tag1-caaaactatcaagtgaactgacagaag
13	APC	15	1st	gaccccactcatgtttagcag	tgccacttaccattccactg
			2nd	Tag1-gaccccactcatgtttagcag	M13-cattccactgcatggttcac
14	APC	15	1st	agtcgttcgattgccagctc	catggtttgtccagggctatc
			2nd	M13-cgattgccagctccgttc	Tag1-catggtttgtccagggctatc
15	APC	15	1st	ccatgcagtggaatggtaag	ggtggaggtgttttacttctgc
			2nd	M13-tggcattataagccccagtg	Tag1-ggtggaggtgttttacttctgc
16	APC	15	1st	gccctggacaaaccatgc	agcagtaggtgctttatttttagg
			2nd	M13-gacaaaccatgccaccaag	Tag1-agcagtaggtgctttatttttagg
17	APC	15	1st	cacctcctcaaacagctcaa	gcagcatttactgcagcttg
			2nd	M13-tcctcaaacagctcaaacca	Tag1-gcagcatttactgcagcttg
18	APC	15	1st	gcagtaaatgctgcagttcagag	tcaatatcatcatcatctgaatcatc
			2nd	M13-cagttcagagggtccaggtt	Tag1-cactcaggctggatgaacaa
19	APC	15	1st	gcctaaagaatcaaatgaaaacc	atcatcatctgaatcatctaataggtc
			2nd	M13-caaatgaaaaccaagagaaagagg	Tag1-atcatcatctgaatcatctaataggtc
20	PIK3CA	9	1st	gcaatttctacacgagatcctct	tccattttagcacttacctgtgac
			2nd	Tag1-gcaatttctacacgagatcctct	M13-cttacctgtgactccatagaaaatc
21	PIK3CA	20	1st	ctgagcaagaggctttggag	tgtgtggaagatccaatcca
			2nd	Tag1-ctgagcaagaggctttggag	M13-tccaatccatttttgttgtcc
22	TP53	5	1st	cgccatggccatctacaag	ctcaccatcgctatctgagc
			2nd	Tag1-tggccatctacaagcagtca	M13-ctcaccatcgctatctgagc
23	TP53	6	1st	taggtctggcccctcctc	cagttgcaaaccagacctca
			2nd	M13-gcccctcctcagcatcttat	Tag1-cagttgcaaaccagacctca
24	TP53	7	1st	aggttggctctgactgtacca	tcttccagtgtgatgatggtg
			2nd	Tag1-aggttggctctgactgtacca	M13-agtgtgatgatggtgaggatg
25	TP53	8	1st	atctactgggacggaacagc	ccctttcttgcggagattc
			2nd	M13-atctactgggacggaacagc	Tag1-cttgcggagattctcttcct
26	KRAS	1	1st	tttattataaggcctgctgaaaatg	tagctgtatcgtcaaggcactc
			2nd	Tag1-tttattataaggcctgctgaaaatg	M13-cgtcaaggcactcttgcc

Tag 1: 5'-tcccgcgaaattaatacgac

M13: 5'-gtaaaacgacggccagt

Quantification of total plasma DNA by quantitative real-time PCR

The amount of total DNA isolated from plasma samples was quantified using a modified version of a human LINE-1 quantitative real-time PCR assay[1]. Three primer sets were designed to amplify differently sized regions within the most abundant consensus region of the human LINE-1 family (79 bp for: 5'-agggacatggatgaaattgg-3'. 79bp rev: 5'-tgagaatatgcggtgtttgg-3'; 97 bp for: 5'-tggcacatatacaccatggaa-3', 97 bp rev: 5'-tgagaatgatggtttccaatttc-3'; 127 bp for: 5'-acttggaaccaacccaaatg-3', 127 bp rev: 5'-tcatccatgtccctacaaagg-3'). PCR was performed in a 25 µl reaction volume consisting of template DNA equal to 2 µl of plasma, 0.5 U of Platinum Taq DNA Polymerase, 1× PCR buffer (see above), 6% (v/v) DMSO, 1 mM of each dNTP, 1:100,000 dilution of SYBR Green I (Invitrogen), and 0.2 µM of each primer. Amplification was carried out in an iCycler (Bio-Rad) using the following cycling conditions: 94°C for 1 min; 2 cycles of 94°C for 10 s, 67°C for 15 s, 70°C for 15 s; 2 cycles of 94°C for 10 s, 64°C for 15 s, 70°C for 15 s, 2 cycles of 94°C for 10 s, 61°C for 15 s, 70°C for 15 s; 35 cycles of 94°C for 10 s, 59°C for 15 s, 70°C for 15 s. Various dilutions of normal human lymphocyte DNA were incorporated in each plate setup to serve as standards. The threshold cycle number was determined using Bio-Rad analysis software with the PCR baseline subtracted. Each quantification was done in duplicate. The total DNA was calculated using the LINE-1 amplicon closest in size to the amplicon being evaluated for mutations (Supplementary Table 3). When the amplicon was equally close to two different LINE-1 amplicons, the average of the values obtained from these two amplicons was used. In control experiments with plasma, we found that the number of genome equivalents assessed by the assay of LINE sequences was highly correlated with the number of genome equivalents (GE) of *APC, KRAS, PIK3CA,* or *RAS* assessed individually by real-time PCR. LINE sequence-based assays, rather than assays of these individual genes, were chosen to measure GE because the former required a much smaller amount of plasma to measure GE as a result of its highly repeated nature in the genome.

BEAMing

Twelve different primer sets were designed for the analysis of 20 mutations (**Supplementary Methods Table 2**). The DNA purified from 2 ml of plasma was used for each BEAMing assay. An initial amplification with a high fidelity DNA polymerase was performed in eight separate 50 µl PCR reactions each containing template DNA from 250 µl of plasma, 5× Phusion High Fidelity PCR buffer (NEB), 1.5 U of Hotstart Phusion polymerase (NEB), 0.2 µM of each primer, 0.25 mM of each dNTP, and 0.5 mM $MgCl_2$. Temperature cycling was carried out as described in the legend to **Supplementary Methods Table 2**. Using the primers also listed in **Supplementary Methods Table 2**, a second PCR (nested) was performed by adding 2 µl of the first amplification to a 20-µl PCR reaction of the same makeup as the first one. PCR products were pooled, diluted, and quantified using the PicoGreen dsDNA assay (Invitrogen). The fluorescence intensity was measured using a CytoFluor multiwell plate reader (PE Biosystems) and the DNA quantity was calculated using Lambdaphage DNA reference standards.

Supplementary Methods Table 2 - Primers used for amplification of plasma DNA.

Amplicon No.	Gene	Exon	Patient No.	PCR	Size (bp)	Forward primer 5'-3'	Reverse primer 5'-3'	PCR Conditions	Size (bp) Line 1 PCR
3	APC	15	17	1st	99	Tag2-tggagagagaacgcggaattggt	Tag1-ctgcagtggtggagatctgcaaac	A	97
5	APC	15	13	1st	116	gtgtagaagatactccaatatgtttttcaagatgtagttc	gtattagcagaatctgcttcctgtgtcg	B	97 & 127
				2nd	108	Tag2-gtgtagaagatactccaatatgtttttcaagatgtagttc	Tag1-cagaatctgcttcctgtgtcgtctg	E	
6	APC	15	14, 16	1st	120	Tag1-ctgaagatgaaataggatgtaatcagac	Tag2-cacaggatcttcagctgacctagttccaatc	A	127
9	APC	15	18	1st	95	Tag2-tgtgagcgaagttccagcagtgtc	Tag1-ctttgtgcctggctgattctgaag	A	97
10	APC	15	2,10	1st	110	aaatccagcagactgcagggttctag	ggtgtctgagcaccacttttggag	A	97 & 127
				2nd	102	Tag2-aaatccagcagactgcagggttctag	Tag1-agcaccacttttggagggagatttc	D	
13	APC	15	6	1st	115	Tag1-caggagaccccactcatgtttagcag	Tag2-cacttaccattccactgcatggttcac	A	97 & 127
16	APC	15	3	1st	98	Tag2-gacaaaccatgccaccaag	Tag1-agcagtaggtgctttatttttagg	C	97
18	APC	15	1,4	1st	111	atgctgcagttcagagggtccag	tcagagcactcaggctggatgaac	A	97 & 127
				2nd	106	Tag2-gcagttcagagggtccaggttcttc	Tag1-tcagagcactcaggctggatgaac	D	
20	PIK3CA	9	5,8	1st	101	gctcaaagcaatttctacacgagatcctct	cagagaatctccattttagcacttacctgtgac	A	97
				2nd	90	Tag1-gctcaaagcaatttctacacgagatcctct	Tag2-cattttagcacttacctgtgactccatagaaaatc	D	
22	TP53	5	9	1st	84	Tag1-tctacaagcagtcacagcacatgacg	Tag2-gctgctcaccatcgctatctgagc	A	79 & 97
25	TP53	8	5,8,11, 16	1st	95	Tag2-tggtaatctactgggacggaacagctt	Tag1-ctttcttgcggagattctcttcctctg	A	97
26	KRAS	1	1,3,4,6, 7,10,12 ,15,17	1st	96	Tag2-tgactgaatataaacttgtggtagttg	Tag1-catattcgtccacaaaatgattc	C	97

Tag1: 5'-tcccgcgaaattaatacgac
Tag2: 5'-gctggagctctgcagcta

A: 98°C 30 s, 37x (98°C 10 s, 71°C 10 s)
B: 98°C 30s, 35 x (98°C 10 s, 69°C 10 s, 72°C 10s)
C: 98°C 30 s, 3 x (98°C 10 s, 70°C 10 s, 72°C 10 s), 3 x (98°C 10 s, 67°C 10 s, 72°C 10 s), 3 x (98°C 10 s, 64°C 10 s, 72°C 10 s), 28 x (98°C 10 s, 61°C 10 s, 72°C 10 s)
D: 98°C 30 s, 4 x (98°C 10 s, 71°C 10 s)
E: 98°C 30 s, 4 x (98°C 10 s, 69°C 10 s, 72°C 10 s)

Emulsion PCR was performed as described previously[2]. Briefly, a 150 µl PCR mixture was prepared containing 18 pg template DNA, 40 U of Platinum Taq DNA polymerase (Invitrogen), 1× PCR buffer (see above), 0.2 mM dNTPs, 5 mM $MgCl_2$, 0.05 µM Tag1 (5'-tcccgcgaaattaatacgac-3'), 8 µM Tag2 (5'-gctggagctctgcagcta-3') and ~$6x10^7$ magnetic streptavidin beads (MyOne, Invitrogen) coated with Tag1 oligonucleotide (5'-dual biotin-T-Spacer18- tcccgcgaaattaatacgac-3'). The 150 µl PCR reaction, 600 µl oil/emulsifier mix (7% ABIL WE09, 20% mineral oil, 73% Tegosoft DEC, Degussa Goldschmidt Chemical), and one 5 mm steel bead (Qiagen) were added to a 96 deep well plate 1.2 ml (Abgene). Emulsions were prepared by shaking the plate in a TissueLyser (Qiagen) for 10 s at 15 Hz and then 7 s at 17 Hz. Emulsions were dispensed into eight PCR wells and temperature cycled at 94°C for 2 min; 3 cycles of 94°C for 10 s, 68°C for 45 s, 70°C for 75 s; 3 cycles of 94°C for 10 s, 65°C for 45 s, 70°C for 75 s, 3 cycles of 94°C for 10 s, 62°C for 45 s, 70°C for 75 s; 50 cycles of 94°C for 10 s, 59°C for 45 s, 70°C for 75 s.

To break the emulsions, 150 µl breaking buffer (10 mM Tris-HCl, pH 7.5, 1% Triton-X 100, 1% SDS, 100 mM NaCl, 1 mM EDTA) was added to each well and mixed with a TissueLyser at 20 Hz for 20 s. Beads were recovered by spinning the suspension at 3,200 g for 2 min and removing the oil phase. The breaking step was repeated twice. All beads from 8 wells were consolidated and washed with 150 µl wash buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). The DNA on the beads was denatured for

5 min with 0.1 M NaOH. Finally, beads were washed with 150 µl wash buffer and resuspended in 150 µl of the same buffer.

The mutation status of DNA bound to beads was determined by allele-specific hybridization. Fluorescently labeled probes complementary to the mutant and wild-type DNA sequences were designed for 20 different mutations. The size of the probes ranged from 15 to 18 nt depending on the GC content of the target region. All mutant probes were coupled to a Cy5™ fluorophore and all wild-type probes were coupled to a Cy3™ fluorophore at their 5' ends (Integrated DNA Technologies or Biomers). In addition, oligonucleotides that bound to a separate location within the amplicon were used to label every extended PCR product as a positive control. These amplicon specific probes were synthesized with a ROX™ fluorophore attached to their 5' ends. Probe sequences are listed in **Supplementary Methods Table 3**. Each allele-specific hybridization reaction contained ~$1x10^7$ beads in 30 µl wash buffer (see above), 66 µl of 1.5× hybridization buffer (4.5 M tetramethylammonium chloride, 75mM Tris-HCl pH 7.5, 6 mM EDTA), and 4 µl of a mixture of mutant, wild-type, and gene-specific fluorescent probes, each at 5 µM in TE buffer. The hybridization mixture was heated to 70°C for 10 s and slowly (0.1°C/s) cooled to 35°C. After incubating at 35°C for 2 min, the mixture was cooled (0.1°C/sec) to room temperature. The beads were collected with a magnet and the supernatant containing the unbound probes was removed using a pipette. The beads were resuspended in 100 µl of 1× hybridization buffer and heated to 48°C for 5 min to remove unbound probes. After the heating step, beads were again separated magnetically and washed once with 100 µl wash buffer. In the final step, the supernatant was removed and beads resuspended in 200 µl TE buffer for flow cytometric analysis.

A LSR II flow cytometry system (BD Bioscience) equipped with a high throughput autosampler was used for the analysis of each bead population. An average of $5×10^6$ beads were analyzed for each plasma sample. Beads with no extension product were excluded from the analysis. Negative controls, performed using DNA from patients without cancer, were included in each assay. Depending on the mutation being queried, the fraction of beads bound to mutant-specific probes in these negative control samples varied from 0.0061% to 0.00023%. This fraction represented sequence errors introduced by the high fidelity DNA polymerase during the first PCR step, as explained in detail previously[3]. To be scored as positive in an experimental sample, (i) the fraction of beads bound to mutant fragments had to be higher than the fraction found in the negative control, and (ii) the mean value of mutant DNA fragments per sample plus one standard deviation had to be >1.0. Bead populations generated by BEAMing were analyzed at least twice for each plasma sample.

References

1. Rago, C. et al. Serial Assessment of Human Tumor Burdens in Mice by the Analysis of Circulating DNA. *Cancer Res* **67**, 9364-9370 (2007).
2. Diehl, F. et al. BEAMing: single-molecule PCR on microparticles in water-in-oil emulsions. *Nat Methods* **3**, 551-9 (2006).
3. Li, M., Diehl, F., Dressman, D., Vogelstein, B. & Kinzler, K.W. BEAMing up for detection and quantification of rare sequence variants. *Nat Methods* **3**, 95-7 (2006).

Supplementary Methods Table 3 - Probes used to discriminate wt from mutant sequences on beads.

Gene	Mutation	Patient No.	Amplicon No.	Probe	Probe Sequence 5'-3'
APC	C2626T	17	3	Universal	ROX-agcaacagaaaatccagga
				Wild-type	Cy3-cttcaaagcgaggtttg
				Mutant	Cy5-cttcaaagtgaggtttg
APC	G3862T	13	5	Universal	ROX-gtagttcattatcatcttt
				Wild-type	Cy3-atgaaataggatgtaatc
				Mutant	Cy5-atgaaatatgatgtaatc
APC	3877-3889 del ACACA GGAAGCAG	14	6	Universal	ROX-tagttccaatcttttctttt
				Wild-type	Cy3-atctgcttcctgtgtcg
				Mutant	Cy5-tagcagaatcgtctgat
APC	3905del T	16	6	Universal	ROX-tagttccaatcttttctttt
				Wild-type	Cy3-ctatttgcagggtatta
				Mutant	Cy5-ctatttgcgggtattag
APC	C4012T	18	9	Universal	ROX-cacagcaccctagaaccaa
				Wild-type	Cy3-agcagactgcagggtt
				Mutant	Cy5-agcagactgtagggtt
APC	C4031A	2	10	Universal	ROX-ttcttcaggagcgaaatct
				Wild-type	Cy3-tttatcttcagaatcagc
				Mutant	Cy5-tttatcttaagaatcagc
APC	C4067A	10	10	Universal	ROX-tagtttatcttcagaatca
				Wild-type	Cy3-attttcttcaggagcga
				Mutant	Cy5-attttcttaaggagcga
APC	G4189T	6	13	Universal	ROX-aactgacagaagtacatct
				Wild-type	Cy3-acgactctcaaaactat
				Mutant	Cy5-acgactctaaaaactat
APC	C4348T	3	16	Universal	ROX-cagaagtaaaacacctcca
				Wild-type	Cy3-aaaccaagcgagaagta
				Mutant	Cy5-aaaccaagtgagaagta
APC	4461delT	1	18	Universal	ROX-tcagagggtccaggttctt
				Wild-type	Cy3-gctgatactttattaca
				Mutant	Cy5-gctgatacttattacat
APC	4465-4468 del TTAC	4	18	Universal	ROX-tcagagggtccaggttctt
				Wild-type	Cy3-tactttattacattttgc
				Mutant	Cy5-gatactttaattttgcca
PIK3CA	G1624A	5, 8	20	Universal	ROX-acctgtgactccatagaaa
				Wild-type	Cy3-agtgatttcagagagag
				Mutant	Cy5-agtgattttagagagag
TP53	C535T	9	22	Universal	ROX-cctcacaacctccgtcatg
				Wild-type	Cy3-gcgctcatggtgggggc
				Mutant	Cy5-gcgctcatagtgggggc
TP53	C817T	11	25	Universal	ROX-cctgggagagaccggcgca
				Wild-type	Cy3-tgaggtgcgtgtttgtg
				Mutant	Cy5-tgaggtgtgtgtttgtg
TP53	G818A	8	25	Universal	ROX-cctgggagagaccggcgca
				Wild-type	Cy3-tgaggtgcgtgtttgtg
				Mutant	Cy5-tgaggtgcatgtttgtg
TP53	C844T	5, 16	25	Universal	ROX-tgaggtgcgtgtttgtgcc
				Wild-type	Cy3-tgagagaccggcgcaca
				Mutant	Cy5-tgagagactggcgcaca
KRAS	G35A	6, 10, 17	26	Universal	ROX-tgacgatacagctaattca
				Wild-type	Cy3-ggagctggtggcgta
				Mutant	Cy5-ggagctgatggcgta
KRAS	G35C	3	26	Universal	ROX-tgacgatacagctaattca
				Wild-type	Cy3-ggagctggtggcgta
				Mutant	Cy5-ggagctgctggcgta
KRAS	G35T	7, 15	26	Universal	ROX-tgacgatacagctaattca
				Wild-type	Cy3-ggagctggtggcgta
				Mutant	Cy5-ggagctgttggcgta
KRAS	G38A	1, 4, 12	26	Universal	ROX-tgacgatacagctaattca
				Wild-type	Cy3-tgctggtggcgtaggc
				Mutant	Cy5-tgctggtgacgtaggc